APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1982, p. 903–908
0099-2240/82/100903-06$02.00/0

Vol. 44, No. 4

Aerosol Stability of Infectious and Potentially Infectious Reovirus Particles

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Received 1 February 1982/Accepted 17 June 1982

The aerosol stability of two particle forms, infectious and potentially infectious, of reovirus were examined under static conditions for a range of relative humidities at 21 and 24°C. Virus aerosolization efficiency was determined for two methods of dissemination: Collison nebulizer and Chicago atomizer. Suspensions of *Bacillus subtilis* var. *niger* spores were added to reovirus preparations that included both particle forms and disseminated into a dynamic aerosol toroid to estimate the physical decay of the aerosols. At 90 to 100% relative humidity, both reovirus particle forms showed less than 10-fold loss of infectivity after 12 h of aging. At lower relative humidities the aerosol decay curve showed rapid initial decay followed by a markedly lower decay rate. Our findings reveal that reovirus particles are relatively stable in the airborne state.

Potential health hazards from bacterial aerosols arising from various agricultural, municipal, and industrial sources have, to some degree, been defined (16, 23). However, the literature indicates that a lack of technology has prevented adequate assessment of viral aerosols generated by these activities. Recent legislation and economic considerations have resulted in increased reuse of polluted waters and sewage effluents. The reuse of polluted waters has increased the potential biohazard from airborne enteric viruses. Aerosols of pathogenic viruses pose an increased threat to health because of the large numbers of enteric viruses in wastewater, their greater resistance to inactivation under conditions which normally eliminate enteric bacteria, and the small number of these viruses potentially capable of infecting humans when inhaled or ingested.

Quantitative detection of any animal virus is difficult, particularly after disinfection of its sources and dilution of the remaining viable virus particles as aerosols in the atmosphere (19; F. K. Fannin, Ph.D. dissertation, University of Michigan, Ann Arbor, 1976). There are relatively small numbers of animal viruses in aerosols naturally produced from polluted waters. These low virus levels require that an appropriate method for concentrating and enumerating animal viruses from aerosol collection fluids be selected. Due to the high dilution of viruses in aerosols, the enteric viruses occurring most frequently and abundantly in contaminated waters should be investigated to assess their potential as indicators of aerosol biohazards.

Reoviruses have been reported to occur in wastewaters and sewage as frequently as picornaviruses (11, 17, 20, 22). Also, rapid and efficient methods for concentrating and assaying reoviruses are available to determine their presence in aerosol collection fluids (11, 18).

Two forms of virus particles are released from reovirus-infected cell cultures, infectious reovirus (IV) and potentially infectious reovirus (PIV). PIV particle forms have a complete outer coat and are not infectious until the outer coat is removed by treatment with proteolytic enzymes (25).

The survival of reoviruses during desiccation varies. Buckland and Tyrrell (7) showed that reoviruses survive being dried well on glass slides at both low and high relative humidities (RHs). Wellings et al. (29) reported finding infectious reovirus in air-dried sludge. In contrast, Ward and Ashley (27) demonstrated significant reductions in reovirus and other enteric virus titers upon dewatering sludge through evaporation.

Our study examines the effect of aerosolization, at various relative humidities, on the stability of combined IV and PIV particle forms.

MATERIALS AND METHODS

Virus and cells. Reovirus type 1 (Lang strain) was used in this study. The virus was produced in mouse L-929 cell suspension cultures maintained in Joklik modified minimal essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 2 to 5% fetal bovine serum (Sterile Systems, Inc., Logan, Utah). After 24 h of infection, the suspension

TABLE 1. Reovirus aerosolization by Collison nebulizer at 21 to 24°C

Reovirus prepn	Prehumidification of secondary air	Particle form	% IFU recovered after aerosolization (± SD)
CsCl gradient centrifugation	No	IV	1.3 ± 0.31
		PIV	5.1 ± 1.21
CsCl gradient centrifugation	Yes	IV	82.1 ± 10.15
		PIV	50.4 ± 10.76
Freon extract	No	IV	1.3 ± 0.36
		PIV	4.9 ± 0.35
Freon extract	Yes	IV	85.3 ± 4.53
		PIV	53.8 ± 4.18

cultures were centrifuged to concentrate the infected cells. Freon 113 (Rakon, Wichita, Kans.) and 6- to 12-ml volumes of infectious tissue culture fluids were used to extract virus from the concentrated cell pellets. These cultures were pooled to form a single lot of reovirus which assayed at approximately 10^{10} immunofluorescent cell-forming units (IFU) per ml. A portion of the Freon 113-extracted virus was further purified by equilibrium centrifugation in a CsCl gradient. Each virus preparation was divided into aliquots and stored at −85°C until needed.

Enzyme treatment and virus assay. Enhancement of the infectivity of reovirus preparations treated with proteolytic enzymes was done by the method of Spendlove and Schaffer (26), except that virus preparations were exposed to 200 μg of trypsin per ml of sample. Assays of IV and PIV were performed before and after the trypsin treatment on Maden-Darby bovine kidney cells. Virus infectivity was assayed with confluent 15-mm cover slip cell cultures inoculated with 0.02 ml of the virus suspension (26). After 20 to 24 h the cell cultures were dried, fixed with cold acetone, and stained with an anti-reovirus fluorescent antibody. The fluorescing cells on two cover slips for each dilution were counted, averaged, and reported as IFU per milliliter.

Physical tracer. Spores of *Bacillus subtilis* var. *niger* (*Bacillus globigii* [BG]) (Bioferm, Wasco, Calif.) were used to determine physical decay rates and recovery ratios of aerosols. The BG stock was used to prepare a working suspension containing 10^{10} spores per ml in Earles balanced salt solution (EBSS; GIBCO), pH 7.2. Samples collected from the dynamic aerosol toroid (DAT) which contained spores were centrifuged for 20 min at 2,000 × *g* to separate the reovirus and BG spores. Spore-containing samples were assayed by plating 0.1-ml quantities of log dilutions, three plates per dilution, on tryptose agar (Difco Laboratories, Detroit, Mich.). Plates were incubated at 37°C for 24 h before bacterial colonies were counted. Average counts are reported.

Aerosol storage equipment. The 1,000-liter stainless steel DAT described by Goldberg et al. (12) and the accompanying mixing chamber were cleaned with live steam and detergent and then rinsed with water. This was followed by six rinses in distilled water, with the final rinse in water containing $NaHCO_3$. Air supplied to the DAT via the mixing chamber or aerosol generator was first passed through coalescing and activated charcoal filters. Aerosols were aged in the 1,000-liter DAT, which was rotating at 4.5 rpm during all phases of the experiment. After each experiment, the drum was air washed at a rate of 120 liters/min for a minimum of 3 h to lower the concentration of virus and BG spores to an acceptable level before the next experiment.

Aerosol generation and sampling. Two aerosol generators were employed: a Collison three-jet nebulizer and a Chicago atomizer. The aerosol generators were operated under conditions which produced particles with a mean diameter of 2.0 μm (Collison nebulizer) or 5 μm (Chicago atomizer). To determine aerosolization efficiencies, we sampled the aerosols generated in the mixing tube during aerosol generation before they entered the DAT. Antifoam (GE 60; General Electric Co., Waterford, N.Y.), was added to a final concentration of 1% to the combined reovirus-BG spore suspension and to the collection fluids. The spray suspension was prepared by mixing equal quantities of BG spore suspension and reovirus stock. All aerosol samples were collected with 6- to 15 all-glass impingers operated for 2 to 5 min at critical vacuum. Each experiment was repeated a minimum of three times and the data are represented by an average decay rate. Samples collected during the aerosol aging experiments were taken from the DAT at 30-min intervals after a 10-min equilibration period at the cessation of aerosol generation.

Aerosol transport media. The efficiency of Collision aerosolization of Freon 113-extracted reovirus particles was analyzed in various transport media at 25 to 35% RH and temperatures of 21 to 24°C. Preliminary experiments showed that similar virus recoveries were obtained when reovirus suspensions prepared with MEM, EBSS, glycine (0.5 M), or standard saline citrate buffer (0.15 M NaCl, 0.015 M sodium citrate, pH 7.2) were aerosolized with BG spores diluted in EBSS. In additional tests, these media were supplemented with one or more of the following: antifoam (1.0% final concentration), fetal bovine serum (0.5 to 2.0%), bovine serum albumin (0.5 to 2.0%), tryptose phosphate broth (0.5 to 2.0%), raffinose (0.5 to 2.0%), or inositol (0.5 to 2.0%). MEM plus 1% antifoam was consistently superior and was used in all subsequent experiments.

RESULTS

Aerosolization efficiency. The aerosolization efficiency of reovirus IV and PIV particles was determined for two methods of dissemination, Collison nebulizer and Chicago atomizer. Reovirus survival (retention of infectivity) after aerosolization with the Collison nebulizer (Table 1) was approximately the same for both Freon 113-extracted reovirus and for virus purified by density gradient centrifugation in CsCl. The IV particles showed an increased survival after aerosolization with prehumidified secondary air (mixing air) of more than 80%, whereas PIV particle survival increased about 50%. Reovirus aerosolized by the Collison nebulizer showed higher survival rates than that aerosolized with

TABLE 2. Reovirus aerosolization by Chicago atomizer at 21 to 24°C

Reovirus prepn	Prehumidification of secondary air	Particle form	% IFU recovered after aerosolization (± SD)
CsCl gradient centrifugation	No	IV	1.2 ± 0.35
		PIV	1.6 ± 0.34
CsCl gradient centrifugation	Yes	IV	11.1 ± 1.75
		PIV	22.9 ± 5.26
Freon extract	No	IV	1.7 ± 0.94
		PIV	2.0 ± 0.97
Freon extract	Yes	IV	13.3 ± 3.96
		PIV	29.4 ± 9.03

TABLE 3. Reovirus decay during equilibration at various RHs at 21 to 24°C[a]

RH range (%)	Particle form	Avg reovirus decay (%/min)	Decay range (%/min)
25–35	IV	3.2	3.0–3.3
	PIV	3.2	3.1–3.3
45–55	IV	2.8	2.5–3.1
	PIV	2.9	2.5–3.1
65–75	IV	3.2	3.1–3.3
	PIV	3.3	3.2–3.3
85–95	IV	2.5	1.7–3.1
	PIV	1.5	0.15–2.1

[a] Average decay rates shown are the decay rates taken from the period after aerosolization until aerosol equilibration (zero time samples) in the DAT.

the Chicago atomizer (Table 2). In each instance (Tables 1 and 2), samples of 2-min duration were collected from the mixing tube before the aerosol entered the DAT, with collection starting 2 min after the initiation of aerosol generation.

With the Chicago atomizer, we again saw that reovirus survival after aerosolization was greatly enhanced by prehumidification of the secondary air. However, the PIV particles showed a greater increase in survival rate. The lower aerosolization efficiencies obtained with the Chicago atomizer were due, at least in part, to impingement of the aerosol spray on the side of the mixing tube.

Reovirus aerosol stability. Duncan's test at the 95% confidence level was used to determine significant differences between experiments in this investigation. All aging studies were performed with reovirus obtained by Freon 113 extraction of cell pellets. The decay kinetics for IV and PIV reovirus particles were biphasic; the particles underwent a rapid initial decay until equilibration in the DAT (Table 3), followed by a markedly lower decay rate upon storage (Fig. 1). The virus decay rates shown (Table 3, Fig. 1 and 2), represent average total decay rates which are composites of the physical decay exhibited by the BG spore and biological decay.

Table 3 shows the percentage of IV or PIV reovirus decay after aerosol generation to the time of equilibration in the DAT. Decay rates ranged from a high of 3.3% per min for both IV and PIV particles at 25 to 35 and 65 to 75% RH to 2.9% per min at 45 to 55% PH. Decay rates for IV and PIV reovirus particles held at 85 to 95% RH were the lowest obtained; the particle decay rates were statistically the same as at 45 to 55% RH. However, PIV particles showed a significantly lower decay rate of 1.5% per min during equilibration at 85 to 95% RH.

At 25 to 35% RH, both IV and PIV particles exhibited similar decay rates after stabilization in the DAT. After storage at 25 to 35 and 45 to 55% RH, IV particles exhibited a slower decay rate than did PIV particles. Survival at 65 to 75% RH was much the same as at 25 to 35% RH. Reovirus IV and PIV particles showed the greatest stability at a range of 85 to 95% RH.

Total decay rates observed after holding the reovirus particles in the DAT varied from 0.6% per min for IV particles held at 45 to 55% RH to 0.3% per min for PIV particles held at 85 to 95% RH over a 2.5-h interval. The overall decay rates for IV and PIV particles were approximately 0.1% per min over a 12-h storage period, indicating that aerosolized reovirus particles can retain infectious properties for extended time periods (Fig. 2). Significant differences were observed among decay rates of airborne reovirus particles stored at each of the four RH ranges examined.

Figure 3 shows the data obtained on reovirus IV and PIV particle infectivity during aerosol storage at 21 to 24°C. Virus survival was a function of RH and time, with strong correlation observed between RH and survival of both IV and PIV reovirus particles. Both reovirus particle forms retained high levels of infectivity throughout the 2.5-h sampling period at both high (65 to 75 and 85 to 95%) and at low (25 to 35%) RHs. However, at the mid-range of 45 to 55% RH, there was a pronounced decline in infectivity with the duration of storage for both reovirus particle forms.

DISCUSSION

Various reports have suggested that the recovery of aerosolized viruses is greatly affected by the transport medium employed in generating the aerosol and by the nature of the collection fluid. Salts have been shown to reduce recovery at intermediate and high RHs (5, 6) but to protect enteric viruses at low RHs (13). The addition of proteins (4, 6, 10) or of almost any compound to a spray fluid may influence the resultant aerosol stability of a virus (5, 13, 14). We found that MEM supplemented with 1%

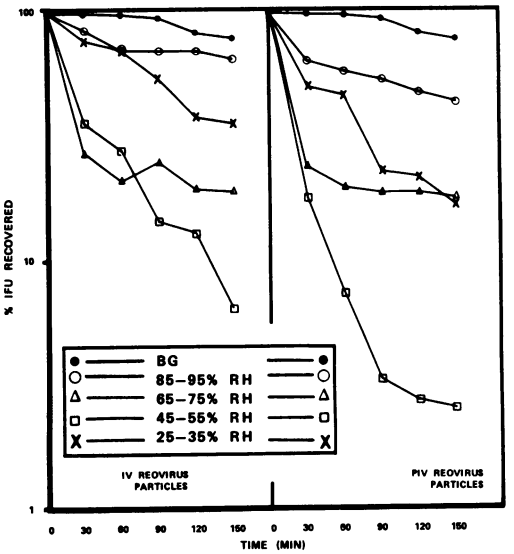

FIG. 1. Total decay rates (physical decay, as shown by BG spores, plus biological decay) of IV and PIV reovirus particles at 21 to 24°C and a range of RHs.

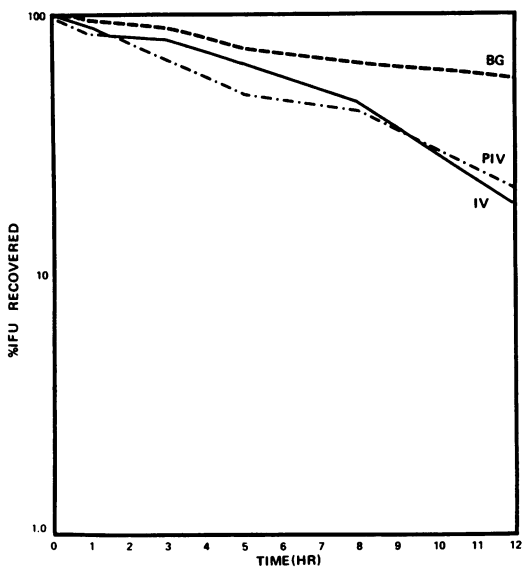

FIG. 2. Decay of IV and PIV reovirus particles held at 21 to 24°C and at 85 to 95% RH for 12 h.

antifoam, a silicone antifoam component, allowed the highest recovery of both IV and PIV reovirus particles. EBSS with 1% antifoam was found to perform well for collection and transport of samples.

Reovirus infectivity immediately after aerosolization by Collison or Chicago generators is

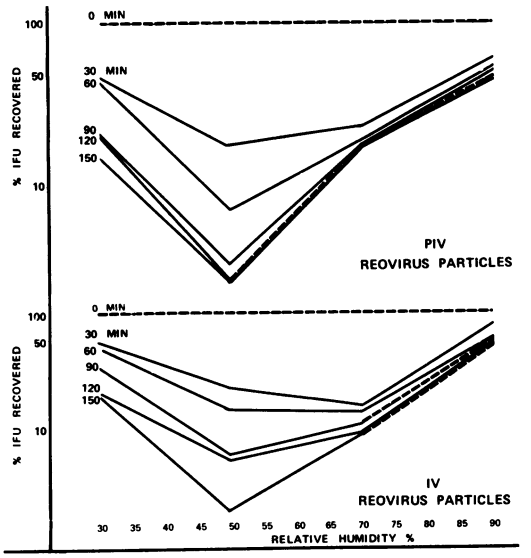

FIG. 3. Effect of RH on survival of airborne IV and PIV reovirus particles at 21 to 24°C.

an indication of virus resistance to the rapid changes taking place during the first phases of equilibration with the test atmosphere. This study demonstrates that prehumidification of the secondary (mixing) air is very important to the survival of reovirus in the DAT. Reovirus recoveries were increased up to 80% upon prehumidification of the secondary air for dissemination by the Collison nebulizer. Aerosol generation by the Chicago atomizer, under conditions which humidified the secondary air, increased recoveries from 11 to 29% for IV and PIV particles, respectively. The difference in increased recovery of IV and PIV particles between the Collison and Chicago atomizers suggests that the difference in method of aerosol generation or particle size of the aerosol produced may effect IV and PIV reovirus particle survival not only during the initial equilibration phase but possibly during storage as well. Impingement of the aerosol spray onto the side of the mixing tube was also an important factor when the Chicago atomizers were used.

Observations by Hatch and Warren (15) suggest that reovirus recovery may have been further increased by prehumidification before sampling with all-glass impingers was made. However, a study by Warren et al. (28) showed that prehumidification before sampling decreased recovery of the animal virus tested.

Samples taken from the bowl of the Collison nebulizer both before and after aerosol generation for periods of up to 5 min showed no significant decrease in IV and PIV titers, indicating that the reflux action used in this method of

aerosol production was not harmful to reovirus particles.

Reovirus particles were exposed to shear forces during aerosol dissemination, desiccation upon equilibration with the ambient air, and decay (both physical and biological) during storage. The reovirus virion is a rigid structure which tends to be resistant to disruption by mechanical forces or by repeated freeze-thaw procedures in aqueous solutions (8). This characteristic, and the increased survival of the virus after prehumidification of the secondary air, suggest that reovirus particles, like other picornaviruses, may be sensitive to desiccation, i.e., during equilibration in the test environment. Our results show that dissemination of reovirus preparations at low RHs produced extensive inactivation, which supports this conclusion. Storage of the reovirus aerosol in the DAT resulted in a much slower rate of inactivation. The decay rates of airborne reovirus particles stored at each of the four RH ranges were significantly different. However, IV particles exhibited slower decay rates than did PIV particles when stored at 25 to 35 and 45 to 55% RH. The decay rates of the two reovirus particle forms were approximately the same at higher RH ranges.

The mechanism of reovirus inactivation during the initial equilibration phase and during storage might first appear to be different, as the inactivation rates are. However, since spray droplets could possibly contain many virus particles, the rapid loss of infectivity upon spraying, especially at lower RH, may represent inactivation of reovirus particles on the outside of the droplet nuclei. A similar mechanism may inactivate the more protected viral particles on the inside of the droplet nuclei more slowly during storage.

It is interesting to note that the inactivation of reovirus particles at various RH ranges is similar to that observed for vesicular stomatitis virus, Rous sarcoma virus, measles virus, polio virus, T3 coliphage, and Col-SK group viruses (2). The infectivity of these viruses when airborne is also RH dependent; infectious virus are consistently recovered at low or high RH, but the particles are rapidly inactivated within the range of 40 to 60% RH.

PIV reovirus particles may have been inactivated in a step-wise manner by being converted to IV particles which are then inactivated. A step-wise inactivation would explain the significantly slower decay rates observed for IV particles at 25 to 35 and 45 to 55% RH. The intact outer coat found in PIV particles may be more difficult to remove with enzyme when it is dried. However, previous studies have indicated that the biological activity of infectious viral nucleic acids was not damaged by aerosol storage (1, 2). This observation suggests that airborne inactivation of reovirus IV and PIV particles may be associated primarily with damage to the virion protein, which in turn may cause a change in the RNA. Work by Akers et al. (3), Dubovi and Akers (10), and DeMik and DeGroot (9) also support this hypothesis. There is also the possibility that the inactivation of reovirus particles, especially at humidities less than 85% RH, is caused by rehydration upon sampling rather than by virus decay during aerosolization or storage.

In summary, microorganisms, once airborne, are exposed to a variety of environmental stresses such as temperature, RH, and UV radiation. This study has shown that at the temperature range and RHs tested, both IV and PIV reovirus particles are relatively stable in the airborne state and may retain infectious properties for extended time periods.

ACKNOWLEDGMENTS

The work upon which this study is based was supported by funds provided by the Office of Water Research and Technology (project no. USDI/OWRT 14-34-0001-8047), U.S. Department of the Interior, by research grant R-806,527 from the Environmental Protection Agency, and by the Department of the Army Project 1T161101A91A In-House Laboratory Independent Research.

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